ARTICLE: DISCOVERIES

Highly expressed genes are preferentially co-opted for C_4 photosynthesis

Jose J. Moreno-Villena[1], Luke T. Dunning[1], Colin P. Osborne[1], Pascal-Antoine Christin[1,2]

[1] Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, United Kingdom.

[2] Corresponding author: Pascal-Antoine Christin, p.christin@sheffield.ac.uk, +44-114-222-0027

Running title: High expression increases co-optability

Keywords: C_4 photosynthesis, evolvability, grasses, phylogenetics, transcriptomics, gene co-option

Abstract

Novel adaptations are generally assembled by co-opting pre-existing genetic components, but the factors dictating the suitability of genes for new functions remain poorly known. In this work, we used comparative transcriptomics to determine the attributes that increased the likelihood of some genes being co-opted for C_4 photosynthesis, a convergent complex trait that boosts productivity in tropical conditions. We show that independent lineages of grasses repeatedly co-opted the gene lineages that were the most highly expressed in non-C_4 ancestors to produce their C_4 pathway. While ancestral abundance in leaves explains which genes were used for the emergence of a C_4 pathway, the tissue specificity has surprisingly no effect. Our results suggest that levels of key genes were elevated during the early diversification of grasses and subsequently repeatedly used to trigger a weak C_4 cycle via relatively few mutations. The abundance of C_4-suitable transcripts therefore facilitated physiological innovation, but the transition to a strong C_4 pathway still involved consequent changes in expression levels, leaf specificity, and coding sequences. The direction and amount of changes required for the strong C_4 pathway depended on the identity of the genes co-opted, so that ancestral gene expression both facilitates adaptive transitions and constrains subsequent evolutionary trajectories.

Introduction

The evolution of novel physiological adaptations occasionally requires the development of new biochemical cascades, which are generally achieved via the co-option of pre-existing genes into new functions (Duboule & Wilkins 1998; True & Carroll 2002; Monson 2003; Monteiro & Podlaha 2009). Rewiring of biochemical pathways can require both modifications of spatial and temporal gene expression patterns and alterations of the coding sequences to adapt the encoded enzymes to the new catalytic context (Duret & Mouchiroud 2000; Carroll 2008; Aubry et al. 2014). In cases where numerous modifications are needed, the novel pathways can be assembled by natural selection only if a functional version can emerge through relatively few changes, allowing subsequent selection to fix mutations that increase the efficiency of the pathway. Genomic factors that reduce the phenotypic distance between ancestral and novel physiologies, thereby enabling the emergence of novel cascades via few mutations, would consequently be expected to increase accessibility to novel phenotypes. However, in most cases these factors remain poorly understood.

The ability of given genes or genomic features to trigger evolutionary innovation can be investigated via experimental evolution (e.g. Weinreich et al. 2006; Blount et at. 2012), but such studies are restricted to short-lived organisms that do not encapsulate the existing diversity of phyla. For larger organisms with long generation times, a historical approach is the most appropriate. Indeed, phylogenetic inference allows explicit tests of how specific features affect the accessibility of new phenotypes (e.g. Marazzi et al. 2012). Conversely, genomic features that have recurrently contributed to independent origins of a given phenotype can be safely assumed to be suitable for the trait of interest, and their origin can be regarded as potentially facilitating later adaptive transitions (Huang et al. 2016b). For example, the same autosome pairs were repeatedly co-opted to evolve sex chromosomes in turtles (Montiel et al. 2017), the same gene families encoding crystallins were used to evolve camera eyes in cephalopods and vertebrates (Zinovieva et al. 1999; Yoshida et al. 2015), and homologous genes recurrently contributed to the diversification of coloration patterns in butterflies (Jiggins et al. 2017). While such evidence indicates that some genomic regions or genes preferentially contribute to specific evolutionary transitions (Tenaillon et al. 2012), multiple factors might increase the adaptive potential, and their identification requires the comparison of the ancestral condition of genes or genomic regions that were recurrently co-opted, to those that were not.

An excellent system to study the factors that increase gene adaptive potential is C_4 photosynthesis. This novel physiology requires a biochemical cascade arising from the high activity of multiple enzymes in specific leaf compartments, and improves autotrophic carbon assimilation in

tropical conditions (Pearcy and Ehleringer, 1984; Hatch 1987; Sage et al., 2012, Atkinson et al. 2016). The C_4 trait is ecologically and agronomically extremely important (Ehleringer et al., 1997; Still et al., 2003; Byrt et al., 2011). It evolved more than 60 times in independent lineages of flowering plants (Sage et al. 2011), via the co-option of multiple genes that were present in non-C_4 ancestors (Hibberd and Quick 2002; Aubry et al. 2011; Brown et al. 2011; Kajala et al. 2012). Most enzymes of the C_4 pathway are encoded by multigene families, whose members differed in their expression patterns and catalytic properties of the encoded enzymes before their involvement in C_4 photosynthesis (Wang et al., 2009; Hibberd and Covshoff, 2010; Aubry et al. 2011; Christin et al., 2013, 2015). Previous comparisons of a handful of C_4 species have shown that a subset of gene lineages were recurrently co-opted for C_4 evolution, both among grasses and among the distantly-related Caryophyllales (Christin et al. 2013, 2015). However, the co-opted genes differed between grasses and Caryophyllales, suggesting that factors predisposing some genes for a C_4 function are specific to subgroups of angiosperms (Christin et al. 2015). It has been noted that the co-opted genes appeared to be highly expressed in the non-C_4 taxa available at the time for comparison, which might have contributed to their preferential co-option (Christin et al. 2013; Emms et al. 2016). However, systematic tests of the factors underlying the observed co-option bias are still lacking.

In this study, we compare transcriptomes across ten independent C_4 origins in grasses, and their non-C_4 relatives. Through a combination of phylogeny-based analyses, we test (i) whether a bias in the gene lineages co-opted exists across the whole set of grasses. To determine the causal factors underlying the bias, we then test (ii) whether the expression level in leaves and/or (iii) whether the tissue specificity in the non-C_4 ancestors explain variation in the co-option probability among gene lineages. In addition, we analyse coding sequences to test (iv) whether adaptive changes in the coding sequences occurred during or after the emergence of the C_4 physiology. Together, our investigations shed new light on the factors that increase the adaptive potential of some genes, focusing on a complex trait of ecological and agronomical importance.

Results

Sequencing, read mapping and transcriptome assembly

In total, 74 individually sequenced RNA libraries from 19 species generated over 550 million 100bp paired-end reads. This represents 98.87 Gb of data, with a mean of 1.34 Gb per library (SD = 0.95 Gb; Table S1). Over 81% of the reads were kept after removing low-quality reads and ribosomal RNA sequences. Transcriptomes were assembled with a mean of 2.23 Gb per species (SD = 1.40

Gb), resulting in a mean of 54,255 Trinity 'unigenes' (SD = 17,218.35), 79,566.12 contigs (SD = 23,038.61), and a 1560.05 bp N50 (SD = 184.95 bp).

The C_4-related gene families considered in this study constitute 5.1% (SD = 2.02%) of the reads in the leaf libraries of C_4 plants, versus 2.34% in non-C_4 plants (SD = 0.75%). On average, 1.05% of the reads from the root libraries mapped to C_4-related genes (SD = 0.48%).

Phylogenetic trees and identification of genes co-opted for C_4 photosynthesis

A total of 533 nuclear core-orthologs were used to infer the species tree, which was well resolved (Fig. 1). The relationships among grass subfamilies mirror those retrieved previously with other datasets (GPWG II, 2012). However, relationships within the Paniceae tribe (the group most densely sampled here) differ in several aspects from those based on plastid markers (GPWG II, 2012), and were closer to previous analyses that also included nuclear markers (Vicentini et al. 2008). The placement of the different C_4 origins within the tree was largely congruent with previous studies, and their non-C_4 relatives separated them in the phylogeny as expected (Fig. 1).

For each gene family encoding C_4-related enzymes, phylogenetic inference confirmed previous conclusions about orthology (Vilella et al. 2009). The enzyme phosphoenolpyruvate carboxykinase (PCK) and the Na^+/H^+ antiporter (NHD) are each encoded by a single gene lineage (Fig. S1). The number of grass co-orthologs in other families varies from two (for pyruvate, phosphate dikinase - PPDK) to eight (for triose phosphate-phosphate translocator – TPT; Fig. S1). Groups of co-orthologs were named as in Christin et al. (2015). Phylogenetic relationships inferred in these gene trees were mostly congruent with the species tree. Exceptions include genes for PCK, where *Echinochloa stagnina* and *Alloteropsis semialata* grouped with those of *Setaria barbata*. This pattern has previously been reported for *Alloteropsis* species and this, together with a number of other lines of evidence, was interpreted as the fingerprint of a lateral gene transfer from *Setaria* or its close relatives (Christin et al. 2012; Dunning et al. 2017). Other incongruences were observed in genes encoding PEPC, PPDK, NAD(P)-malate dehydrogenase [NAD(P)-MDH], Sodium bile acid symporter family (SBAS), TPT, and NDH (Fig. S1), and could stem from a combination of reticulate evolution during grass diversification and phylogenetic bias due to adaptive evolution. Gene duplicates specific to subgroups of grasses are evident for several genes, and can in some cases be associated to recent polyploidy (e.g. in *Zea mays* genes *pck-1P1*, *ppc-1P4*, *ppdk-1P2*, *nadmdh-4P7*; Fig. S1). Our analytical pipeline cannot estimate the expression level individually for each of these duplicates with very similar sequences, but these duplications specific to subgroups of grasses are relatively recent and occurred after the divergence of C_3 and C_4 clades (Fig. S1). The

inferred evolutionary changes in expression patterns and co-option events are consequently not affected.

The most highly transcribed genes encoding C_4-related proteins are those for β-carbonic anhydrase (βCA; Fig. 2; Table S2), an enzyme that acts in the cytosol of mesophyll cells in C_4 plants. These genes are however equally abundant in non-C_4 species (Fig. 2), where the enzyme plays a key role in the chloroplasts of mesophyll cells (Tetu et al. 2007). Of the 31 other gene families encoding enzymes that can be related to the C_4 pathway, 14 included gene lineages with transcript abundances above 500 rpkm in at least one C_4 species (Fig. 3; Table S2). The transcript abundance of *ppa-4P4* reached 500 rpkm in some C_4 species, but similar abundance was observed in a number of non-C_4 taxa (Table S2), and the gene was consequently not counted as C_4 specific. For the rest of the gene lineages, such high values were not found in non-C_4 species (Table S2). Genes co-opted for C_4 photosynthesis were identified in each C_4 species for most core C_4 enzymes, but putative C_4 transporters and regulators were not always abundant in C_4 leaves (Table S2). Genes for enzymes of the photorespiration pathway were downregulated in C_4 species, as expected (Table S2).

Factors affecting gene co-option

Out of 58 gene lineages encoding the 14 enzymes used by the C_4 species sampled here, only 18 have been co-opted at least once, and up to ten times independently for *ppdk-1P2* and *tpt-1P1* and eight for *ppc−1P3* (Table 1). Given the size of the different gene families and the number of co-option events, fewer genes have been co-opted at least once than expected by chance (p-value < 0.00001). This confirms the existence of a co-option bias across the ten C_4 origins considered here, a result previously reported for Caryophylalles and grasses (Christin et al. 2013, 2015).

The ancestral state reconstructions inferred the abundance in leaves and leaf/root specificity in the last common ancestor of the sampled grasses for each C_4-related (Fig. 4). This approach comes with uncertainty, especially for deeper nodes in a tree, but the confidence intervals associated with the inferred values are small compared to the difference among members of the same gene family (Fig. 4). The inferred values are moreover tightly correlated with averages of the values among C_3 grasses (R^2 = 0.98 for the leaf abundance and R^2 = 0.91 for the leaf/root ratio), and were consequently used for modelling of gene co-option. Linear models showed that the ancestral transcript abundance in the leaf significantly affected the co-option frequency (F=13.11, df=56, p=0.0006336; R^2=0.19), and this stayed significant when the gene family was used as a co-factor (Table 2). The effect of the ancestral leaf/root transcript abundance ratio on the co-option frequency was not significant when considered on its own (F=0.40, df=56, p=0.54), or in combination with the

ancestral leaf abundance and the gene family cofactor (Table 2). Therefore, our modelling analyses indicate that genes were co-opted for C_4 photosynthesis based on their transcription level in leaves (Fig. 4), independently of the specificity of this expression in leaves compared with roots. The same conclusions were reached when using a threshold of 300, 1000 and 1500 rpkm for the identification of co-opted genes (see Table 2).

Transcriptome datasets for clades containing C_3 and C_4 species other than grasses are focused on small taxonomic groups, so that ancient evolutionary events cannot be inferred yet outside from grasses. A test using published transcriptomes for one C_3 and C_4 species within the eudicot family Cleomaceae failed to detect any effect of expression levels, on the identity of genes co-opted for C_4 (Tables S3 and S4), but the availability of a single C_4 origin and only one C_3 relative likely decreased statistical power. Although the same statistical limitations applied to the *Flaveria* dataset, our preliminary investigation suggested that the effect of leaf abundance on the co-option probability might apply to multiple C_4 origins across the angiosperms. Indeed, there was a significant effect of the leaf abundance in the close relatives on the co-option probability for *Flaveria* (Table S4).

Marked differences in transcript abundance and coding sequences

While the ancestral transcript abundance significantly affects the probability of a gene being co-opted, the evolution of C_4 photosynthesis is accompanied by major increases in transcript abundance. The transcripts of genes encoding C_4 enzymes increase by a fold change of up to 480 for *ppc-1P6* in *Alloteropsis semialata* compared to related non-C_4 taxa (Fig. 2). In addition, their leaf specificity increases, to reach leaf/root ratios of up to 6204 after their co-option into C_4 photosynthesis, compared to a maximum of 257 in non-C_4 taxa (Fig. 3).

Besides these changes in transcript abundance, tests for positive selection revealed adaptive evolution in the coding sequences of a number of genes during or slightly after their co-option into C_4 photosynthesis. After correction for multiple testing, the test for a shift of selective pressures along C_4 branches (A1 vs. M1a comparison) was significant for nine genes out of 19 (Table S5). The test specifically testing for a shift to positive selection as opposed to a relaxation of selection (A1 vs. A comparison) was also significant for four of these nine genes; *ppc-1P3*, *ppdk-1P2*, *sbas-1P1*, *and tpt-1P1* (Table S5). The sites identified by the Bayes Empirical Bayes analysis as being under positive selection along C_4 branches showed widespread cases of parallel amino acid replacements (Fig. 5).

Discussion

Expression patterns determined which genes were co-opted for C_4

In this study, we analyzed root and leaf transcriptomes from grass species representing ten independent origins of C_4 photosynthesis as well as the close non-C_4 relatives to each of them (Fig. 1). As previously suggested based on smaller species samples (Christin et al. 2013, 2015), the co-option of genes for the C_4 pathway has been a non-random process. Indeed, despite multiple gene lineages existing for most C_4-related enzymes, a few of them were co-opted more frequently than expected by chance, while most were never used in the ten C_4 lineages evaluated here (Table 1; Fig. 3 and 4). A number of factors could explain the preferential co-option of some genes for a novel function, including their availability via genomic redundancy, the suitability of their kinetic properties, the fit of their expression patterns, and their evolvability (Aharoni et al. 2005; Landry et al. 2007; Christin et al. 2010, 2015; Stiffler et al. 2015; Huang et al. 2016b). Our approach was specifically designed to test for the effects on co-option probability of two dimensions of the expression patterns inferred for non-C_4 ancestors; the transcript abundance in leaves and the leaf versus root specificity. Thanks to the evolutionary-informed sampling (Fig. 1), we were able to unambiguously show that the likelihood of gene co-option into C_4 photosynthesis was determined in a large part by their transcript abundance in leaves prior to C_4 evolution (Fig. 4), with no apparent effect of the leaf to root specificity (Table 2).

The C_4 biochemical pathway, like any complex pathway, is assumed to result from many rounds of fixation of adaptive mutations (Sage et al. 2012; Heckmann et al. 2013; Dunning et al. 2017). However, natural selection cannot gradually improve a pathway before it exists, even in a rudimentary stage (Huang et al. 2016b). It is likely that a primitive, weak C_4 cycle initially emerged in some species via a slight upregulation of few genes, as observed in intermediate plants accumulating only part of their CO_2 via the C_4 cycle (Mallmann et al. 2014; Dunning et al. 2017). We show here for the first time that some genes were already moderately abundant in leaves of non-C_4 plants (Fig. 4), a pattern that likely evolved for a number of reasons not related to C_4 photosynthesis, but eased its later evolution. This facilitator effect would have been even stronger if C_4-related genes were upregulated in the low-CO_2 conditions that prevailed until the Industrial Revolution, as has been suggested for the distantly-related *Arabidopsis* (Li et al. 2014). The encoded enzymes, present in the leaves of the non-C_4 ancestors, constituted the building blocks needed to generate a weak, yet functional, C_4 pathway following key mutations. These could have included further upregulation of key C_4 enzymes or alterations of the leaf structural arrangements, pushing the system beyond a tipping point where the C_4 pathway could emerge. Models predict that,

once a C_4 pathway is in place, any increase in the rate of the C_4 pathway will increase productivity in warm conditions (Heckmann et al. 2013; Mallmann et al. 2014). Any rudimentary C_4 pathway based on ancestrally abundant enzymes would therefore have created the selective impetus for upregulation of enzymes, generating the striking patterns observed in derived C_4 plants (Fig. 2 and 3).

Besides elevated abundance of numerous enzymes, the C_4 trait is characterized by a precise compartmentalization of the biochemical reactions in different parts of the leaves (Hatch and Osmond 1976; Hatch 1987; John et al. 2014). Interestingly, transcript abundance in non-photosynthetic tissues, such as roots, did however not prevent the co-option of a gene lineage for C_4 photosynthesis (Table 2; Fig. 3), and previous pairwise comparisons have established that orthologs to C_4 genes have a diversity of expression patterns in non-C_4 species (Külahoglu et al. 2014). We conclude that being abundant in leaves was a sufficient condition for the C_4 function, independently of the presence in other tissues. Cellular and subcellular localization, which was not captured by our whole-leaf transcriptomes, probably still contributed to determining which genes were co-opted for C_4. For instance, only one of the four gene lineages for NADP-ME present in grasses encodes a chloroplast-specific isoform, and this gene lineage has been recurrently co-opted for C_4 despite an ancestral abundance of a second gene (Fig. 4; Christin et al. 2009). Similarly, the product of *ppc-1P2*, the most highly expressed gene for PEPC in non-C4 plants (Fig. 4), is chloroplast-specific (Masumoto et al. 2010), which very likely prevented a function in C_4 photosynthesis, since this enzyme is cytosolic in the C_4 pathway. Independently of these specific cases, the mere moderate abundance in leaves explains a large fraction of the co-option probability.

Despite genetic enablers, C_4 evolution required massive changes

Our study is the first to scan the transcriptomes of a number of non-C_4 grasses closely related to C_4 species, and showed that genes co-opted for C_4 tended to already be abundant in non-C_4 ancestors (Fig. 3 and 4). Although transcriptomes in other groups are not available for multiple C_4 origins and their C_3 relatives, our reanalysis of eudicot datasets suggested that the preferential co-option of the most abundant genes might underly C_4 origins in groups other than grasses (Table S4). This suggests that the abundance of some enzymes able to fulfil a C_4 function facilitated the emergence of a C_4 pathway. However, massive changes in gene expression are still observed between non-C_4 and C_4 relatives (e.g. Bräutigam et al. 2011, 2014; Külahoglu et al. 2014). Indeed, genes encoding C_4 enzymes are orders of magnitude more abundant in C_4 leaves, and leaf specificity strongly increased after the co-option of genes for C_4 (Fig. 2 and 3). In addition, evidence for widespread adaptive evolution of coding sequences for the C_4 context, obtained here and in other studies (Fig.

5; Besnard et al. 2009; Christin et al. 2009; Wang et al. 2009; Huang et al. 2016a), suggests important modifications of the kinetic properties, shown for some enzymes (Bläsing et al. 2000; Tausta et al. 2002). Instead of being involved in the initial emergence of a C_4 cycle, we propose that these massive changes were involved in the transition from a weak to a strong C_4 pathway able to match the high rates of the Calvin cycle, as suggested for specific study systems (Svensson et al. 2003; Mallmann et al. 2014; Dunning et al. 2017).

Since the major requirement for a C_4 function was sufficient abundance in leaves, the co-opted genes were not necessarily the best suited for the C_4 function, in terms of the tissue specificity or kinetic properties of the encoded enzyme. The ancestral abundance might therefore have constrained the initial emergence of a weak C_4 cycle based on specific sets of genes, forcing natural selection to later adapt their properties to those required for a high-flux strong C_4 cycle. The recurrent co-option of the same co-orthologs would have increased the likelihood of adaptation via similar changes, explaining the observed parallel amino acid replacements among C_4 origins in grasses (Fig. 5; Christin et al. 2007). It has been shown that C_4 lineages belonging to distant groups of angiosperms in some cases co-opted distinct genes (Christin et al. 2015; Table S4). Because of the large evolutionary distances separating these groups, which are further increased when different co-orthologs are co-opted (Table S4), the encoded enzymes likely varied in their kinetic properties in addition to their leaf and cell specificities. The amount of optimizing adaptive changes might have varied among major C_4 groups as a consequence, explaining that the frequency and identity of selection-driven amino acid replacements shows high convergence among closely related C_4 lineages (Fig. 5), but varies between C_4 origins in grasses and those in the distantly related sedges and eudicots (Besnard et al. 2009).

Conclusions

In this study, we sequenced the transcriptomes of species from the main C_4 grass lineages as well as their close non-C_4 relatives, and used models to show that the identity of genes co-opted for C_4 photosynthesis was largely explained by transcript abundance before C_4 evolution. The co-option, likely dictated by the mere presence of each protein in leaves, was followed by massive upregulation and widespread adaptation of coding sequences. Both of these processes likely accelerated and optimized a C_4 pathway that initially emerged from the combined action of enzymes already present in leaves. It is currently unknown why some gene lineages came to be more expressed than others in non-C_4 plants but, despite variation among species, the increased abundance of these genes seems to date back to at least the last common ancestor of grasses.

Comparison among distant groups of angiosperms indicates that the preferential co-option of the most abundant gene lineages might be a recurrent pattern, but the sampling is not yet dense enough across angiosperms to precisely determine when increased transcript abundance first happened, among the ancestors of grasses and other groups that recurrently evolved C_4 photosynthesis. When this information is available, we might be able to test whether gene abundance combined with anatomical variation determined which plant lineages were more likely to evolve C_4 photosynthesis, once environmental changes created the selective pressure for this physiological novelty.

Material and Methods

Species sampling

Grass species were selected for analyses based on their photosynthetic type to include multiple C_4 origins and their non-C_4 relatives, based on previous phylogenetic analyses (GPWG II 2012). We sequenced eight C_4 species and eleven non-C_4 species, which separate them in the phylogenetic tree of grasses (GPWG II 2012, Fig. 1). Most of these belong to the PACMAD clade (subfamilies Panicoideae, Arundinoideae, Chloridoideae, Micraioideae, Aristidoideae and Danthonioideae), which contains all C_4 origins in grasses, and one non-C_4 Pooideae species was added as an outgroup for comparisons.

The selected species were grown from seeds, using the material from Atkinson et al. (2016) and Lundgren et al. (2015). Plants were maintained in controlled environment growth chambers (Conviron BDR16; Manitoba, Canada), with 60% relative humidity, 500 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD), and 25/20°C day/night temperatures, with a 14-hour photoperiod. John Innes No. 2 potting compost (John Innes Manufacturers Association, Reading, England) was used. Plants were watered three times a week to keep the soil damp, and were fertilised every two weeks with Scotts Evergreen Lawn Food (The Scotts Company, Surrey, England). After a minimum of 30 days in these controlled conditions, two young roots and the most photosynthetically active distal half of fully expanded leaves were sampled from two individuals of each species (biological replicates) during the middle of the photoperiod, and immediately frozen in liquid nitrogen. All samples were stored at -80 °C until RNA extraction.

RNA extraction, sequencing and transcriptome assembly

Samples were homogenised in liquid nitrogen using a pestle and a mortar, and RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The isolated RNA was DNA digested on-column using the RNase-Free Dnase Set

(Qiagen, Hilden, Germany) and eluted in RNAse-free water with 20 U/µL of SUPERase-IN RNase Inhibitor (Life Technologies, Carlsbad, CA). Extractions that yielded an RNA integrity number (RIN) greater than 6.5 and at least 0.5 µg of total RNA, as determined with the RNA 6000 Nano kit with an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California), were used for upstream procedures. Individual RNA libraries were prepared using TruSeq RNA Library Preparation Kit v2 (Illumina, San Diego, CA), following the manufacturer's protocol with a target median insert length of 155 bp. A total of 24 indexed libraries were pooled per lane of flow cell and sequenced on an Illumina HiSeq 2500 platform with 100 cycles in rapid mode generating 100bp paired-end reads, at the Sheffield Diagnostic Genetics Service.

Reads were filtered and assembled using the Agalma pipeline version 0.5.0, with default parameters (Dunn *et al.,* 2013). This pipeline removes low quality reads (Q <33), and those that are adaptor-contaminated or correspond to ribosomal RNA. The filtered reads are then used for *de novo* assembly using Trinity (version trinityrnaseq_r20140413p1; Grabherr *et al.*, 2011). One assembly was generated per species, using all the libraries available. Leaf assembly and reads in duplicates from the C_4 *Alloteropsis cimicina* were retrieved from Dunning et al. (2017), and reads for the C_4 *Megathyrsus maximus* and the non-C_4 *Dichanthelium clandestinum*, in triplicates and without replicate, respectively, were retrieved from Bräutigam et al. (2014). RNA-seq reads for C_4 grasses with a completely sequenced genome were also retrieved from the literature [*Setaria italica* without replicate from Zhang et al. (2012), *Zea mays* without replicate from Liu *et al.* (2015), and *Sorghum bicolor* in duplicates from Fracasso *et al.* (2016)]. The final RNA expression dataset included 12 non-C_4 species and 13 C_4 species of grasses.

Inference of a species tree based on core orthologs

Coding sequences (CDS) were predicted from the assembled contigs and those retrieved from the literature using the standalone version of OrfPredictor (Min et al. 2005). Protein sequences of eight publicly available genomes (*Arabidopsis thaliana*, *Brachypodium distachyon*, *Glycine max*, *Oryza sativa*, *Populus trichocarpa*, *Setaria italica*, *Sorghum bicolor* and *Zea mays*) were used as references to improve the identification of open reading frames by providing the program with a pre-computed BLASTX output file, using parameters suggested by the authors (Min et al. 2005). CDS from contigs with "no hit" in the BLASTX output were predicted *ab initio*. The predicted CDS were used for subsequent analyses.

CDS homologous to an *a priori* defined set of plant genes were retrieved using a Hidden Markov Model based search tool (HaMSTR v.13.2.3; Ebersberger et al. 2009). The set of genes includes 581 single copy core-orthologs from plants and is derived from the Inparanoid ortholog

database (Sonnhammer and Ostlund 2014), using five high quality genomes (*Arabidopsis thaliana*, *Vitis vinifera*, *Oryza sativa*, *Sorghum bicolor* and *Ostreococcus lucimarinus*). Sequences were aligned as described in Dunning et al. (2017); alignments shorter than 100 bp after trimming were discarded, and alignments including sequences from at least ten species were concatenated. The resulting alignment was used to infer a maximum likelihood tree with Phyml (Guindon and Gascuel 2003), using a GTR + G + I nucleotide substitution model, which was identified as the best model using the Smart Model Selection (Lefort et al. 2017). Support was evaluated by 100 bootstrap pseudoreplicates.

Identification of homologs and grass co-orthologs encoding C_4-related enzymes

For each gene family that encodes enzymes related to the C_4 pathway (identified based on the literature; Mallmann *et al.*, 2014; Li *et al.*, 2015), homologous CDS were retrieved from three publicly available genomes (*Setaria italica, Sorghum bicolor and Arabidopsis thaliana*), based on the annotation and previously inferred homology (Vilella *et al.*, 2009). The same approach was used to analyse genes of the photorespiration pathway, which are expected to be downregulated during C_4 evolution (Mallmann et al 2014). CDS from the sequenced transcriptomes or retrieved from the literature that were homologous to any sequence in each gene family were identified via BLAST searches. Positive matches with a minimal e-value of 0.01 and minimal mapping length of 500bp were retrieved and added to the datasets. Only the first transcript model was considered for complete genomes, and the longest CDS from each set of Trinity gene isoforms was used.

A new alignment was produced for each gene family ensuring high quality alignments while maintaining as many sites as possible. This approach requires manual curation, and was consequently not used for the 581 sets of core orthologs described above. A preliminary alignment was obtained for each gene family using MUSCLE (Edgar 2004). The alignment was manually inspected in MEGA version 6 (Tamura *et al.* 2013), and potential chimeras and sequences of ambiguous homology (false positives) identified through visual inspection and comparison with other sequences were removed. The remaining sequences were re-aligned as codons using ClustalW (Thompson et al. 1994), and the alignments were manually refined. For each gene family, the alignment was used to compute a maximum likelihood phylogenetic tree, using PhyML (Guindon & Gascuel, 2003), and the GTR + G + I substitution model as best-fit model identified previously for most of the gene families in this study (Christin et al. 2015). Support values were evaluated with 100 bootstrap pseudoreplicates.

Groups of grass co-orthologs, which include all the genes that descend from a single gene in the last common ancestor of grasses through speciation and gene or genome duplications (including

the ancient polyploidy in the common ancestor of grasses; Tang et al. 2010), were identified based on the phylogenetic trees inferred for each gene family. Duplicates specific to some groups of grasses, which might have emerged via gene or genome duplication (whether via auto- or allopolyploidy) after the diversification of grasses, would be grouped in the same co-orthologs, so that our orthology assessment and subsequent expression analyses are not influenced by polyploidization events. Cleaned reads were mapped back to sequences belonging to any of the gene families as single reads, using the local alignment option in Bowtie2 (Langmead & Salzberg, 2012). Our approach allows reads to map back to sequences from the same species, but also allows sequences from other closely related species to serve as the reference. The number of reads mapped to each group of co-orthologs was reported as reads per kilobase of aligned exons per million of cleaned reads (rpkm). These proxies for transcript abundances were obtained for each replicate.

Identification of co-opted genes and factors increasing co-option rates

Enzymes of the C_4 pathway are abundant in the leaves of C_4 species because high catalytic rates are needed to match the fluxes of the Calvin cycle (Furbank et al. 1997, Mallmann et al. 2014). Transcripts encoding enzymes known to act in the C_4 pathway were consequently identified as those that reached an abundance of at least 500 rpkm in leaves of a given C_4 species. Because this threshold is arbitrary, subsequent analyses were repeated with other thresholds (300, 1000, 1500 rpkm), which did not affect our conclusions (see Results). Previous investigations comparing a limited number of species have shown that, within a given taxonomic group, independent C_4 origins tend to co-opt the same gene lineages (Christin et al. 2013, 2015; Emms et al. 2016). To test this expectation across our larger species sample, the number of genes co-opted at least once in our dataset was compared to the number expected by chance given the size of the different gene lineages and the number of co-option events, following the resampling approach of Christin et al. (2015).

Once a bias in gene co-option was confirmed (see Results), we tested for factors potentially affecting the probability of a given group of co-orthologs being co-opted for C_4. We used the values inferred for the last common ancestor of grasses as proxies for the condition before C_4 evolved, with two different dimensions of the expression patterns. First, we inferred the leaf transcript abundance. Second, we inferred the leaf/root ratio of abundances as a proxy for leaf specificity. For each group of co-orthologs, the values of these variables in the common ancestor of grasses were estimated using the phylogeny obtained with HaMSTR and the 'ace' function in the R package ‘ape’ version 3.5 (Paradis et al. 2004). The maximum likelihood method was selected, with a Brownian motion model. In this approach, the value of the continuous variable that maximizes the likelihood is

calculated for each node, with the associated confidence intervals. Only non-C_4 species were included in the ancestral state analyses to avoid biases caused by high levels in C_4 taxa. Considering only the gene families co-opted at least once, linear models, as implemented in the 'lm' function in R version 3.3.2 (R Development Core Team 2016), were used to test independently for an effect of ancestral leaf transcription abundance and of ancestral leaf/root ratio on the number of times each group of co-orthologs has been co-opted. An analysis of variance on multiple linear models was then used to determine whether the effect of ancestral leaf abundance and/or leaf/root ratio remain when the gene family was included as a co-factor.

Transcriptome datasets available for groups of closely related C_3 and C_4 species outside of grasses were used to assess whether the observed patterns are valid across flowering plants. Data for one C_3 and one C_4 Cleomaceae were retrieved from Bräutigam et al. (2011), and the phylogenetic annotation of C_4-related genes in these datasets was deduced from the identity of orthologs from the closely-related *Arabidopsis* and the phylogenetic trees from Christin et al. (2015). For *Flaveria*, RNAseq data were retrieved for two C_3 species from Mallmann et al. (2014) and for one C_4 species from Lyu et al. (2015). The reads were annotated in the original study based on their similarity to *Arabidopsis* sequences, but the evolutionary distance between *Flaveria* and *Arabidopsis* can potentially mislead orthology assessments. We consequently performed *de novo* assemblies using the published reads, and obtained the transcript abundance for C_4-related genes using the previously published phylogenetic annotation pipeline (Christin et al. 2015). Groups of co-orthologs co-opted for C_4 by *Flaveria* or Cleomaceae were identified based on the literature (reviewed in Christin et al. 2015) or based on leaf abundance reaching 500 rpkm in C_4 species for the genes not included in previous reviews. The effect of the abundance in the C_3 relatives on the co-option probability was modelled as for grasses, independently for Cleomaceae and *Flaveria*. Because two C3 species are available for Flaveria, their average abundance was used. Root abundance was not available for the same species, so that the effect of leaf specificity in these groups of eudicots could not be tested.

Positive selection tests

Codon models were used to test for positive selection following the co-option of genes for C_4 photosynthesis. For each group of co-orthologs that has been co-opted at least once for C_4, the inferred alignment was truncated as needed to remove poorly aligning ends and a new phylogenetic tree was inferred with phyML, considering only 3rd positions of codons to remove potential biases due to adaptive evolution. The inferred topology was used to optimize three different codon models, using codeml as implemented in PAML (Yang 2007). These models rely on the ratio of non-synonymous mutation rate per synonymous mutation rate (ω; Yang and Nielsen 2002, 2008; Yang

and Swanson 2002). In the null model M1a, codons evolve under either purifying or relaxed selection in all branches (ω smaller than and equal to one, respectively). In the branch-site models, some codons still evolve under neutral or purifying selection in all branches, but others shift from purifying or relaxed selection in background branches to relaxed (in model A) or positive (in model A1) selection in foreground branches. These foreground branches are defined *a priori*. In our case, all branches descending from each C_4 co-opted gene (identified above for the species sequenced here and from the literature for the rest of species) were set as the foreground branches. Because genes for β-carbonic anhydrase (βCA) were present at similar abundance in non-C_4 and C_4 species (see Results), but these are known to be part of the C_4 pathway (Budde et al.,1985; Hatch and Burnell, 1990), all branches leading to C_4 species in these gene families were selected as foreground branches. The fit improvement of the model assuming changes in selection pressures was evaluated using likelihood ratio tests (LRT). The model A1 was first compared to the model M1a, to test for selective shifts following the co-option event, and then to the model A to specifically test whether the shift corresponded to positive selection. P-values were corrected for multiple testing.

Acknowledgements

This work was supported by the Royal Society (grant numbers RG130448, URF120119), and the Natural Environment Research Council (grant number NE/M00208X/1). All sequences generated in this work have been submitted to NCBI Sequence Read Archive and Transcriptome Shotgun Assembly repository (BioProject PRJNA395007).

References

Aharoni A, Gaidukov L, Khersonsky O, Gould SM, Roodveldt C, Tawfik DS. 2005. The 'evolvability' of promiscuous protein functions. Nature Genet 37:73-76.

Atkinson RRL, Mockford EJ, Bennett C, Christin PA, Spriggs EL, Freckleton RP, Thompson K, Rees M, Osborne CP. 2016. C_4 photosynthesis boosts growth by altering physiology, allocation and size. Nature Plants 2:16038.

Aubry S, Brown NJ, Hibberd JM. 2011. The role of proteins in C_3 plants prior to their recruitment into the C_4 pathway. J Exp Bot 62:3049–3059.

Aubry S, Kelly S, Kümpers BMC, Smith-Unna RD, Hibberd JM. 2014. Deep evolutionary comparison of gene expression identifies parallel recruitment of trans-factors in two independent origins of C_4 photosynthesis. PLoS Genet 10:e1004365.

Bergthorsson U, Andersson DI, Roth JR. 2007. Ohno's dilemma: evolution of new genes under continuous selection. Proc Natl Acad Sci USA 104:17004–17009.

Besnard G, Muasya AM, Russier F, Roalson EH, Salamin N, Christin PA. 2009. Phylogenomics of C_4 photosynthesis in sedges (Cyperaceae): Multiple appearances and genetic convergence. Mol Biol Evol 26:1909–1919.

Bläsing OE, Westhoff P, Svensson P. 2000. Evolution of C_4 phosphoenolpyruvate carboxylase in Flaveria-a conserved serine residue in the carboxyterminal part of the enzyme is a major determinant for C_4-specific characteristics. J Biol Chem 275:27917–27923.

Blount ZD, Barrick JE, Davidson CJ, Lenski RE. 2012. Genomic analysis of a key innovation in an experimental *Escherichia coli* population. Nature 489:513–518.

Bräutigam A, Kajala K, Wullenweber J, Sommer M, Gagneul D, Weber KL, Carr KM, Gowik U, Mass J, Lercher MJ, et al. 2011. An mRNA blueprint for C_4 photosynthesis derived from comparative transcriptomics of closely related C_3 and C_4 species. Plant Physiol 155:142–156.

Bräutigam A, Schliesky S, Külahoglu C, Osborne CP, Weber APM. 2014. Towards an integrative model of C_4 photosynthetic subtypes: insights from comparative transcriptome analysis of NAD-ME, NADP-ME, and PEP-CK C_4 species. J Exp Bot, 65:3579–93.

Brown NJ, Newell CA, Stanley S, Chen JE, Perrin AJ, Kajala K, Hibberd JM. 2011. Independent and parallel recruitment of preexisting mechanisms underlying C_4 photosynthesis. Science 331:1436–1439.

Budde RJA, Holbrook GP, Chollet R. 1985. Studies on the dark/light regulation of maize leaf pyruvate, orthophosphate dikinase by reversible phosphorylation. Arch Biochem Biophys 242:283–290.

Byrt CS, Grof CPL, Furbank RT. 2011. C_4 plants as biofuel feedstocks: Optimising biomass production and feedstock quality from a lignocellulosic perspective. J Integr Plant Biol 53:120–135.

Carroll SB. 2008. Evo-Devo and an expanding evolutionary synthesis: A genetic theory of morphological evolution. Cell 134:25–36.

Christin PA, Arakaki M, Osborne CP, Edwards EJ. 2015. Genetic enablers underlying the clustered evolutionary origins of C_4 photosynthesis in angiosperms. Mol Biol Evol 32:846–858.

Christin PA, Boxall SF, Gregory R, Edwards EJ, Hartwell J, Osborne CP. 2013. Parallel recruitment of multiple genes into C_4 photosynthesis. Genome Biol Evol 5:2174–2187.

Christin PA, Edwards EJ, Besnard G, Boxall SF, Gregory R, Kellogg EA, Hartwell J, Osborne CP 2012. Adaptive evolution of C_4 photosynthesis through recurrent lateral gene transfer. Curr Biol 22:445–499.

Christin PA, Weinreich DM, Besnard G. 2010. Causes and evolutionary significance of genetic convergence. Trends Genet 26:400-405.

Christin PA, Samaritani E, Petitpierre B, Salamin N, Besnard G. 2009. Evolutionary insights on C_4 photosynthetic subtypes in grasses from genomics and phylogenetics. Genome Biol Evol 1:221-230.

Duboule D, Wilkins AS. 1998. The evolution of “bricolage”. Trends Genet 14:54–9.

Dunn CW, Howison M, Zapata F. 2013. Agalma: an automated phylogenomics workflow. BMC Bioinfo 14:330.

Dunning LT, Lundgren MR, Moreno-Villena JJ, Namaganda M, Edwards EJ, Nosil P, Osborne CP, Christin PA. 2017. Introgression and repeated co-option facilitated the recurrent emergence of C_4 photosynthesis among close relatives. Evolution 71:1541–1555.

Duret L, Mouchiroud D. 2000. Determinants of substitution rates in mammalian genes: Expression pattern affects selection intensity but not mutation rate. Mol Biol Evol 17:68–70.

Ebersberger I, Strauss S, von Haeseler A. 2009. HaMStR: Profile hidden markov model based search for orthologs in ESTs. BMC Evol Biol 9:157.

Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797.

Ehleringer JR, Cerling TE, Helliker BR. 1997. C_4 photosynthesis, atmospheric CO_2, and climate. Oecologia 112:285–299.

Emms DM, Covshoff S, Hibberd JM, Kelly S. 2016. Independent and parallel evolution of new genes by gene duplication in two origins of C_4 photosynthesis provides new insight into the mechanism of phloem loading in C_4 species. Mol Biol Evol 33:1796–806.

Fracasso A, Trindade LM, Amaducci S. 2016. Drought stress tolerance strategies revealed by RNA-Seq in two sorghum genotypes with contrasting WUE. BMC Plant Biol 16:115.

Furbank RT, Chitty JA, Jenkins CLD, Taylor WC, Trevanion SJ, von Caemmerer S, Ashton AR. 1997. Genetic manipulation of key photosynthetic enzymes in the C_4 plant *Flaveria bidentis*. Aus J Plant Physiol 24:477-485.

Gowik U, Bräutigam A, Weber KL, Weber AP, Westhoff P. 2011. Evolution of C_4 photosynthesis in the genus *Flaveria*: how many and which genes does it take to make C_4? Plant Cell 23:2087-2105.

GPWGII – Grass Phylogeny Working Group II. 2012. New grass phylogeny resolves deep evolutionary relationships and discovers C_4 origins. New Phytol 193:304-312.

Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, Adiconis X, Fan L, Raychowdhury R, Zeng Q, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnol 29:644–652.

Guindon S, Gascuel O. 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst Biol 52:696–704.

Hatch MD. 1987. C_4 photosynthesis: a unique blend of modified biochemistry, anatomy and ultrastructure. Biochim Biophys Acta 895:81–106.

Hatch MD, Osmond CB. 1976. Compartmentation and transport in C_4 photosynthesis. In: Stocking CR, Heber U, editors. Transport in Plants III. Berlin, Heidelberg: Springer Berlin Heidelberg. p. 144–184.

Hatch MD, Burnell JN. 1990. Carbonic anhydrase activity in leaves and its role in the first step of C_4 photosynthesis. Plant Physiol 93:825-828.

Heckmann D, Schulze S, Denton A, Gowik U, Westhoff P, Weber APM, Lercher MJ. 2013. Predicting C_4 photosynthesis evolution: Modular, individually adaptive steps on a Mount Fuji fitness landscape. Cell 153:1579–1588.

Hibberd JM, Quick WP. 2002. Characteristics of C_4 photosynthesis in stems and petioles of C_3 flowering plants. Nature 415:451–454.

Hibberd JM, Covshoff S. 2010. The regulation of gene expression required for C_4 photosynthesis. Annu Rev Plant Biol 61:181–207.

Huang P, Studer AJ, Schnable JC, Kellogg EA, Brutnell TP. 2016a. Cross species selection scans identify components of C_4 photosynthesis in the grasses. J Exp Bot 68:127-135.

Huang R, O'Donnell AJ, Barboline JJ, Barkman TJ. 2016b. Convergent evolution of caffeine in plants by co-option of exapted ancestral enzymes. Proc Natl Acad Sci USA 113:10613-10618.

Jiggins CD, Wallbank RWR, Hanly JJ. 2017. Waiting in the wings: what can we learn about gene co-option from the diversification of butterfly wing patterns? Philos Trans R Soc Lond B Biol Sci 372:20150485.

John CR, Smith-Unna RD, Woodfield H, Covshoff S, Hibberd JM. 2014. Evolutionary convergence of cell-specific gene expression in independent lineages of C_4 grasses. Plant Physiol 165:62-75.

Kajala K, Brown NJ, Williams BP, Borrill P, Taylor LE, Hibberd JM. 2012. Multiple *Arabidopsis* genes primed for recruitment into C_4 photosynthesis. Plant J 69:47-56.

Külahoglu C, Denton AK, Sommer M, Mass J, Schliesky S, Wrobel TJ, Berckmans B, Gongora-Castillo E, Buell CR, Simon R, et al. 2014. Comparative transcriptome atlases reveal altered gene expression modules between two Cleomaceae C_3 and C_4 plant species. Plant Cell 26:3243-3260.

Landry CR, Lemos B, Rifkin SA, Dickinson WJ, Hartl DL. 2007. Genetic properties influencing the evolvability of gene expression. Science 317:118-121.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nature Methods 9:357–359.

Lefort V, Longueville JE, Gascuel O. 2017. SMS: Smart Model Selection in PhyML. Mol Biol Evol 34:2422-2424.

Li Y, Xu J, Ul Haq N, Zhang H, Zhu XG. 2014. Was low CO_2 a driving force for C_4 evolution: *Arabidopsis* responses to long-term low CO_2 stress. J Exp Bot 65: 3657-3667.

Li Y, Ma X, Zhao J, Xu J, Shi J, Zhu XG, Zhao Y, Zhang H. 2015. Developmental genetic mechanisms of C_4 syndrome based on transcriptome analysis of C_3 cotyledons and C_4 assimilating shoots in *Haloxylon ammodendron*. Plos One. 10:e0117175.

Liu Y, Zhou M, Gao Z, Ren W, Yang F, He H, Zhao J. 2015. RNA-Seq analysis reveals MAPKKK family members related to drought tolerance in maize. Plos One 10:e0143128.

Lyu MA, Gowik U, Kelly S, Covshoff S, Mallmann J, Westhoff P, Hibberd JM, Stata M, Sage RF, Lu H, Wei X, Wong GK and Zhu X. 2015. RNA-Seq based phylogeny recapitulates previous phylogeny of the genus *Flaveria* (Asteraceae) with some modifications. BMC Evolutionary Biology 15:116.

Lundgren MR, Besnard G, Ripley BS, Lehmann CE, Chatelet DS, Kynast RG, Namaganda M, Vorontsova MS, Hall RC, Elia J, Osborne CP, Christin PA. 2015. Photosynthetic innovation broadens the niche within a single species. Ecol Lett 10:1021-1029.

Mallmann J, Heckmann D, Bräutigam A, Lercher MJ, Weber APM, Westhoff P, Gowik U. 2014. The role of photorespiration during the evolution of C_4 photosynthesis in the genus *Flaveria*. Elife 3:e02478.

Marazzi B, Ané C, Simon MF, Delgado-Salinas A, Luckow M, Sanderson MJ. 2012. Locating evolutionary precursors on a phylogenetic tree. Evolution 66:3918–3930.

Masumoto C, Miyazawa SI, Ohkawa H, Fukuda T, Taniguchi Y, Murayama S, Kusano M, Saito K, Fukayama H, Miyao M. 2010. Phosphoenolpyruvate carboxylase intrinsically located in the chloroplast of rice plays a crucial role in ammonium assimilation. Proc Natl Acad Sci USA 107:5226–31.

Min XJ, Butler G, Storms R, Tsang A. 2005. OrfPredictor: predicting protein-coding regions in EST-derived sequences. Nucleic Acids Res 33:W677-W680.

Monson RK. 2003. Gene duplication, neofunctionalization, and the evolution of C_4 photosynthesis. Int J Plant Sci 164:S43–S54.

Monteiro A, Podlaha O. 2009. Wings, horns, and butterfly eyespots:How do complex traits evolve? Plos Biol 7:0209–0216.

Montiel EE, Badenhorst D, Tamplin J, Burke RL, Valenzuela N. 2017. Discovery of the youngest sex chromosomes reveals first case of convergent co-option of ancestral autosomes in turtles. Chromosoma 126:105–113.

Paradis E, Claude J, Strimmer K. 2004. APE: Analyses of Phylogenetics and Evolution in R language. Bioinformatics 20:289–290.

Pearcy RW, Ehleringer J. 1984. Comparative ecophysiology of C_3 and C_4 plants. Plant Cell Environ 7:1–13.

R Development Core Team. 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/.

Sage RF, Christin PA, Edwards EJ. 2011. The C_4 plant lineages of planet Earth. J Exp Bot 62:3155–69.

Sage RF, Sage TL, Kocacinar F. 2012. Photorespiration and the evolution of C_4 photosynthesis. Annu Rev Plant Biol 63:19–47.

Sonnhammer EL, Koonin EV. 2002. Orthology, paralogy and proposed classification for paralog subtypes. Trends Genet 18:619–620.

Stiffler MA, Hekstra DR, Ranganathan R. 2015. Evolvability as a function of purifying selection in TEM-1 β-lactamase. Cell 160:882-892.

Still CJ, Berry JA, Collatz GJ, DeFries RS. 2003. Global distribution of C_3 and C_4 vegetation: Carbon cycle implications. Glob Biogeochem Cycles 17:6–1–6–14.

Svensson P, Bläsing OE, Westhoff P. 2003. Evolution of C_4 phosphoenolpyruvate carboxylase. Arch Biochem Biophys 414:180-188.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30:2725–2729.

Tang H, Bowers JE, Wang X, Paterson AH. 2010. Angiosperm genome comparisons reveal early polyploidy in the monocot lineage. Proc Natl Acad Sci USA 107: 472-477.

Tausta SL, Miller Coyle H, Rothermel B, Stiefel V, Nelson T. 2002. Maize C_4 and non-C_4 NADP-dependent malic enzymes are encoded by distinct genes derived from a plastid-localized ancestor. Plant Mol Biol 50:635–652.

Tenaillon O, Rodríguez-Verdugo A, Gaut RL, McDonald P, Bennett AF, Long AD, Gaut BS. 2012. The molecular diversity of adaptive convergence. Science 335:457-461.

Tetu SG, Tanz SK, Vella N, Burnell JN, Ludwig M. 2007. The *Flaveria bidentis* beta-carbonic anhydrase gene family encodes cytosolic and chloroplastic isoforms demonstrating distinct organ-specific expression patterns. Plant Physiol 144:1316–27.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

True JR, Carroll SB. 2002. Gene Co-Option in Physiological and Morphological Evolution. Annu Rev Cell Dev Biol 18:53–80.

Vicentini A, Barber JC, Aliscioni SS, Giussani LM, Kellogg EA. 2008. The age of the grasses and clusters of origins of C_4 photosynthesis. Global Change Biol 14:2963–2977.

Vilella AJ, Severin J, Ureta-Vidal A, Heng L, Durbin R, Birney E. 2009. EnsemblCompara GeneTrees: Complete, duplication-aware phylogenetic trees in vertebrates. Genome Res 19:327–35.

Wang X, Gowik U, Tang H, Bowers JE, Westhoff P, Paterson AH. 2009. Comparative genomic analysis of C_4 photosynthetic pathway evolution in grasses. Genome Biol 10:R68.

Weinreich DM, Delaney NF, Depristo MA, Hartl DL. 2006. Darwinian evolution can follow only very few mutational paths to fitter proteins. Science 312:111–114.

Yang Z. 2007. PAML 4: Phylogenetic Analysis by Maximum Likelihood. Mol Biol Evol 24:1586–1591.

Yang Z, Nielsen R. 2002. Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. Mol Biol Evol 19:908–917.

Yang Z, Nielsen R. 2008. Mutation-selection models of codon substitution and their use to estimate selective strengths on codon usage. Mol Biol Evol 25:568–579.

Yang Z, Swanson WJ. 2002. Codon-substitution models to detect adaptive evolution that account for heterogeneous selective pressures among site classes. Mol Biol Evol 19:49–57.

Yoshida M, Yura K, Ogura A, Furuya H. 2015. Cephalopod eye evolution was modulated by the acquisition of Pax-6 splicing variants. Sci Rep 4:4256.

Zhang G, Liu X, Quan Z, Cheng S, Xu X, Pan S, Xie M, Zeng P, Yue Z, Wang W, et al. 2012. Genome sequence of foxtail millet (Setaria italica) provides insights into grass evolution and biofuel potential. Nature Biotechnol 30:549–554.

Zinovieva RD, Piatigorsky J, Tomarev SI. 1999. O-Crystallin, arginine kinase and ferritin from the octopus lens. Biochim Biophys Acta 1431:512–517.

Figure captions

Figure 1. Phylogenetic tree based on nuclear orthologs.

C_4 taxa are in red, and C_4 origins are numbered. One of the tribe and the two main clades of grasses are indicated on the right. The black circle highlights the node representing the common ancestor of the sampled grasses. Bootstrap values are indicated near branches.

Figure 2. Transcript abundances of the main C_4 genes in C_4 and non-C_4 species.

Barplot indicate rpkm values (reads per kilobase per million of reads) in leaves of C_4 (in red) and non-C_4 (in black species). Phylogenetic relationships among species are indicated at the top, and C_4 lineages are numbered as in Fig. 1. Species names are abbreviated as in Tables S1 and S2.

Figure 3. Gene expression profiles of C_4-related genes in the studied taxa.

Colors indicate leaf transcript abundance and leaf/ratio abundance ratio for C_4-related genes in C_4 and non-C_4 species. Genes that have been co-opted at least once are at the top.

Figure 4. Ancestral leaf transcript abundance and number of co-option events.

Barplots on the left indicate the number of times each gene was co-opted, and those on the right indicate the inferred abundance in the non-C_4 last common ancestor of grasses (see Fig. 1), with the associated confidence intervals. Genes are sorted by enyzme, indicated on the left.

Figure 5. Patterns of convergent adaptive amino acid replacements.

The phylogeny of the sampled species is indicated on the left, with species names abbreviated as in Table S1. Branches leading to C_4 species in red. Amino acids at sites under positive selection ($p<0.05$*; $p<0.01$**) are indicated on the right. Residues of co-opted genes are highlighted with a blue background.

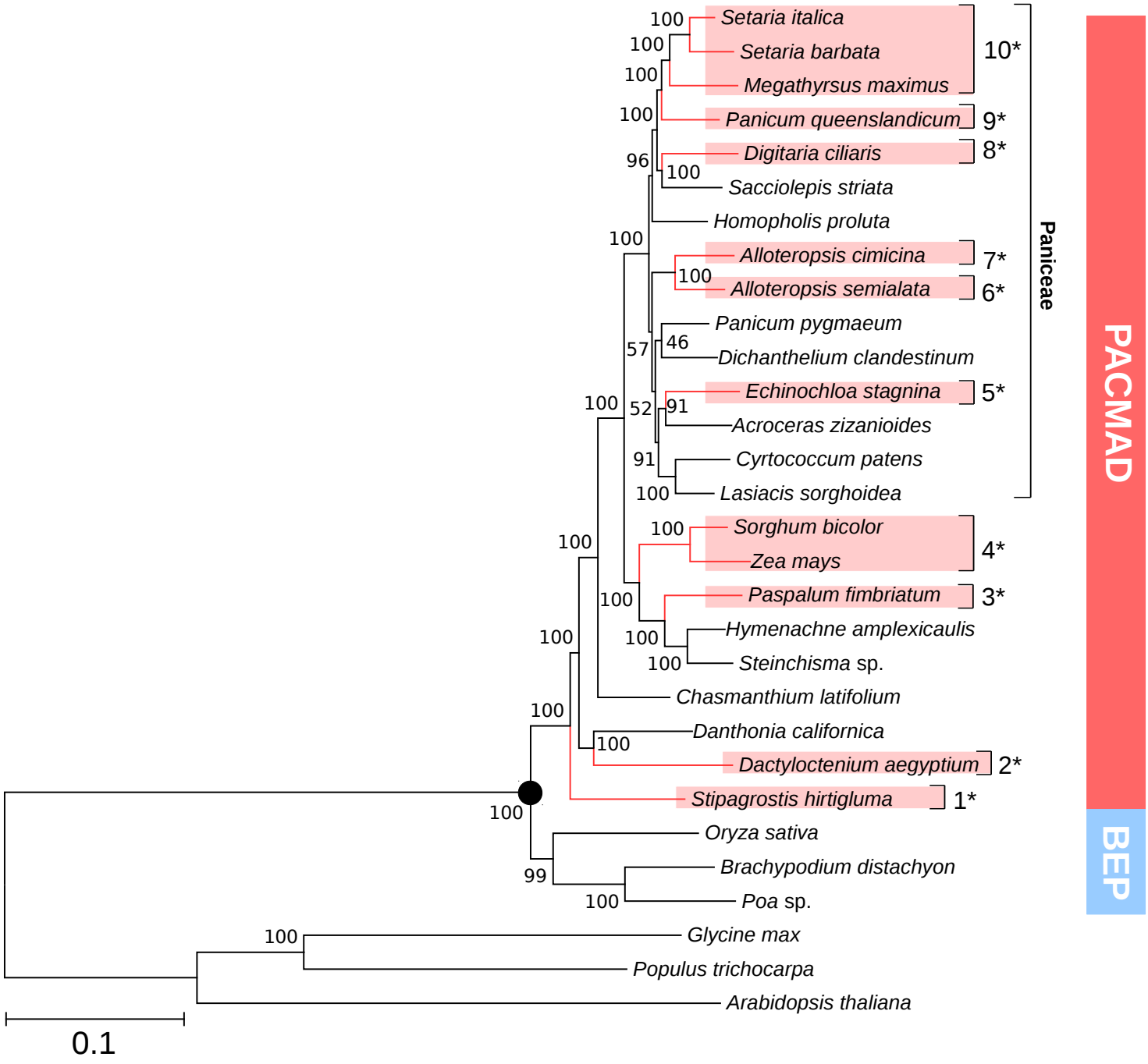
Table 1. Number of times a gene lineage was co-opted, for genes co-opted at least once.

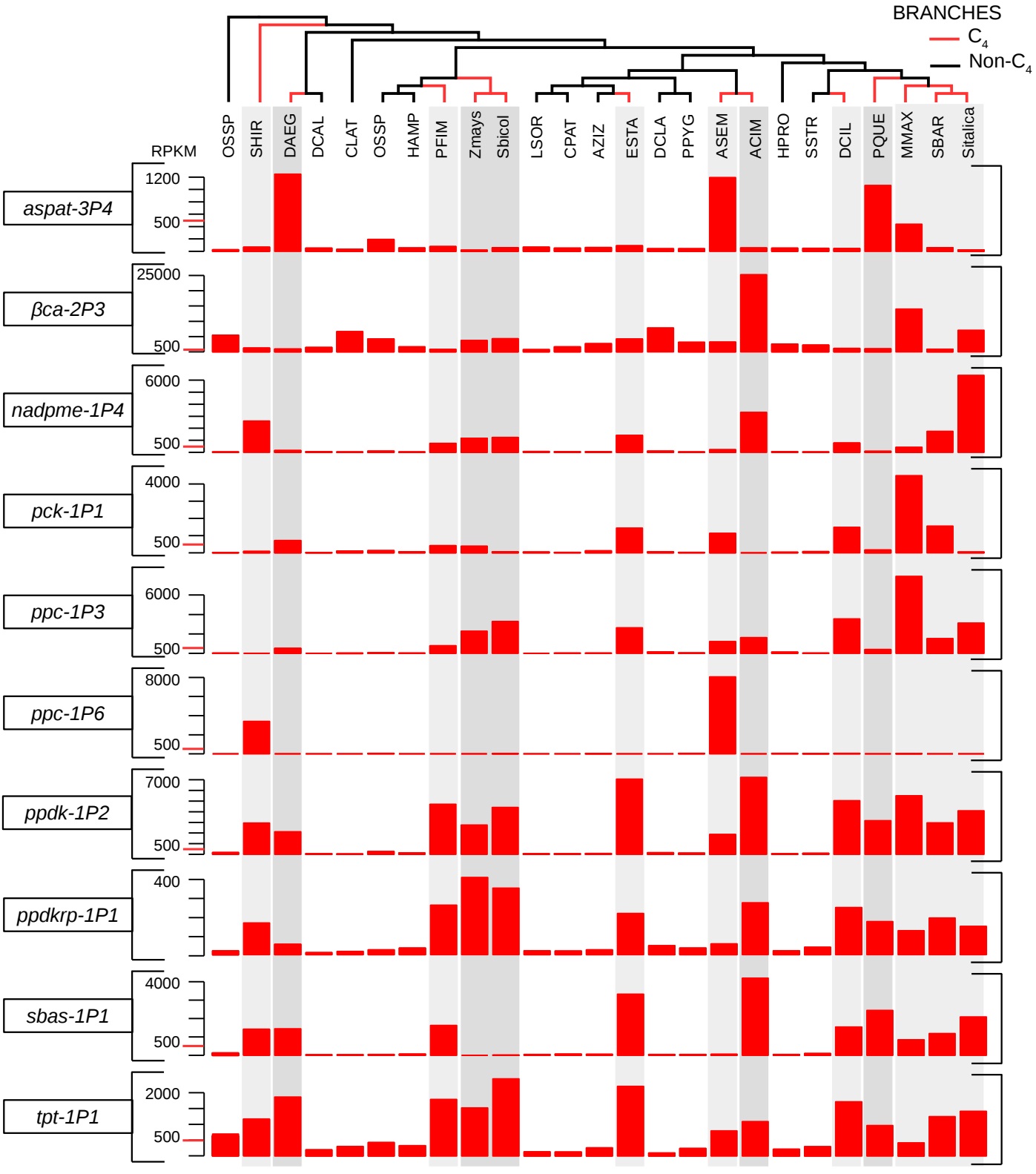
Gene lineage	Times co-opted	Main catalytic reaction
ak-1P1	8	AMP → ADP
alaat-1P5	3	Ala ↔ Pyruvate
aspat-2P3	3	Asp ↔ OAA
aspat-3P4	3	Asp ↔ OAA
dit-2P3	1	Dicarboxylate transporter
nadpmdh-1P1	5	Malate ↔ OAA
nadpmdh-3P4	1	Malate ↔ OAA
nadpme-1P4	7	Malate → pyruvate
nhd-1P1	5	Sodium proton antiport
pck-1P1	5	OAA → PEP
pepck-1P1	1	ATP ADP/P antiport
ppa-1P2.1	6	Pyrophosphate → phosphate
ppc-1P3	8	PEP → OAA
ppc-1P6	2	PEP → OAA
ppdk-1P2	10	Pyruvate → PEP
ppt-1P5	4	PEP phosphate antiport
sbas-1P1	8	Pyruvate sodium symport
tpt-1P1	10	3-PGA TP antiport

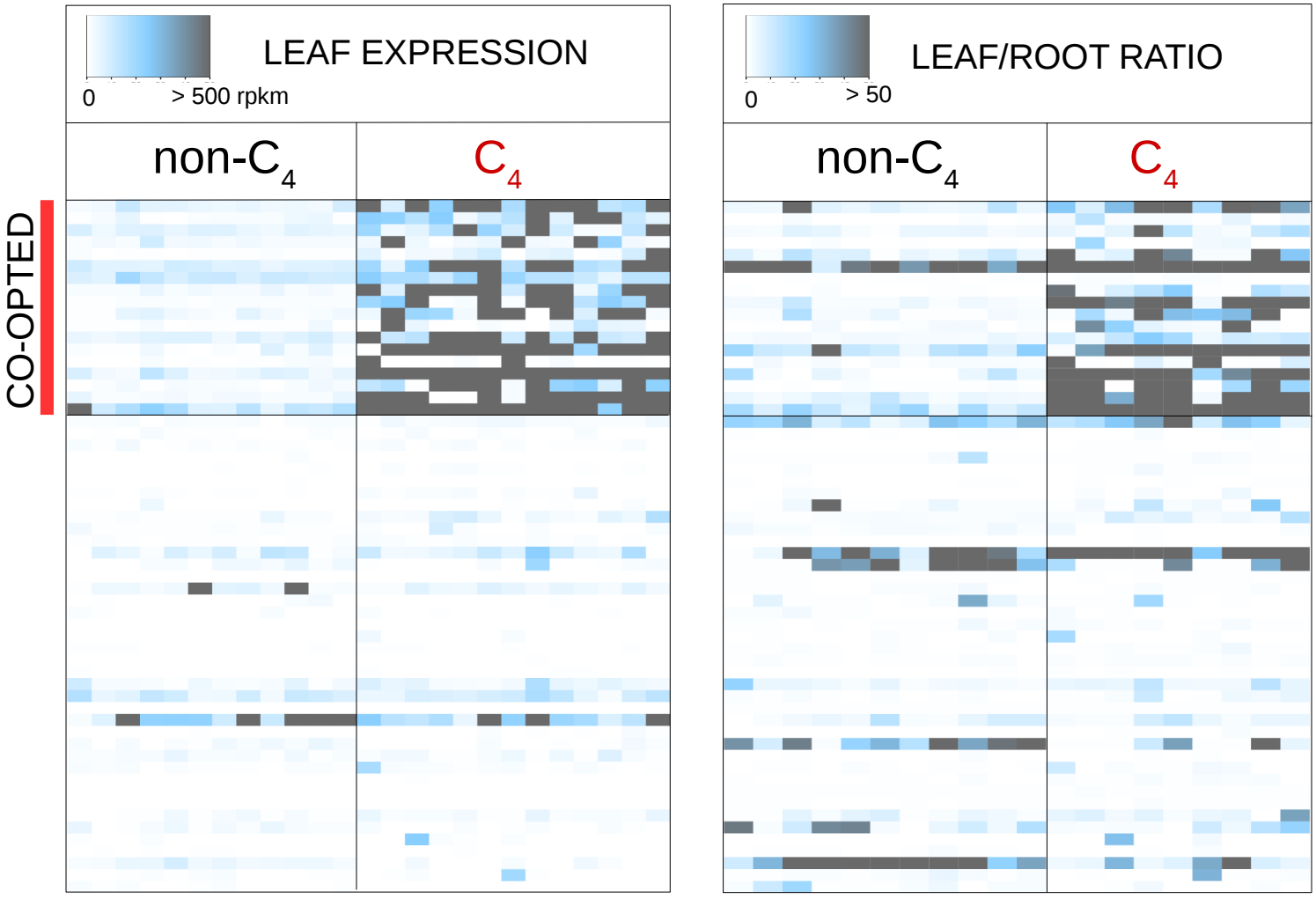
Table 2. Results of analyses of variance on linear models of number of co-option events based on ancestral leaf abundance (ala), leaf/root ratio, and gene family identity (family), with co-opted genes identified with different rpkm thresholds.

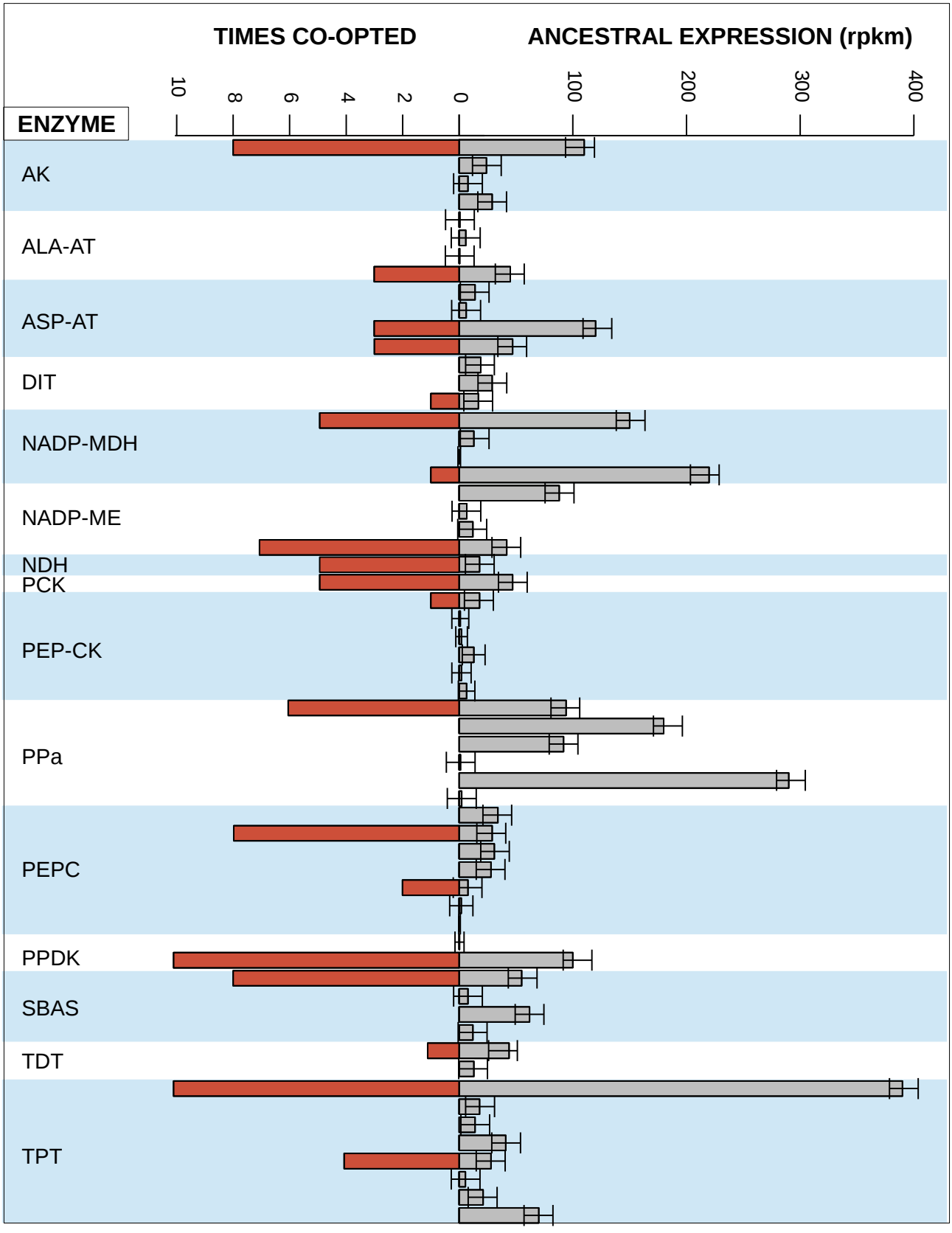
rpkm threshold	**300**	**300**	**300**	**500**	**500**	**500**	**1000**	**1000**	**1000**	**1500**	**1500**	**1500**
Factors	**ala**	**leaf/root**	**family**	**ala**	**leaf/root**	**family**	**ala**	**leaf/root**	**family**	**ala**	**leaf/root**	**family**
p-value	0.00	0.52	0.38	0.00	0.57	0.56	0.00	0.88	0.21	0.01	0.77	0.10
df[1]	1,42	1,42	13,42	1,42	1,42	13,42	1,42	1,42	13,42	1,42	1,42	13,42
F-stat	17.07	0.78	0.95	12.65	0.32	0.90	14.46	0.21	1.37	8.29	0.0.09	1.71

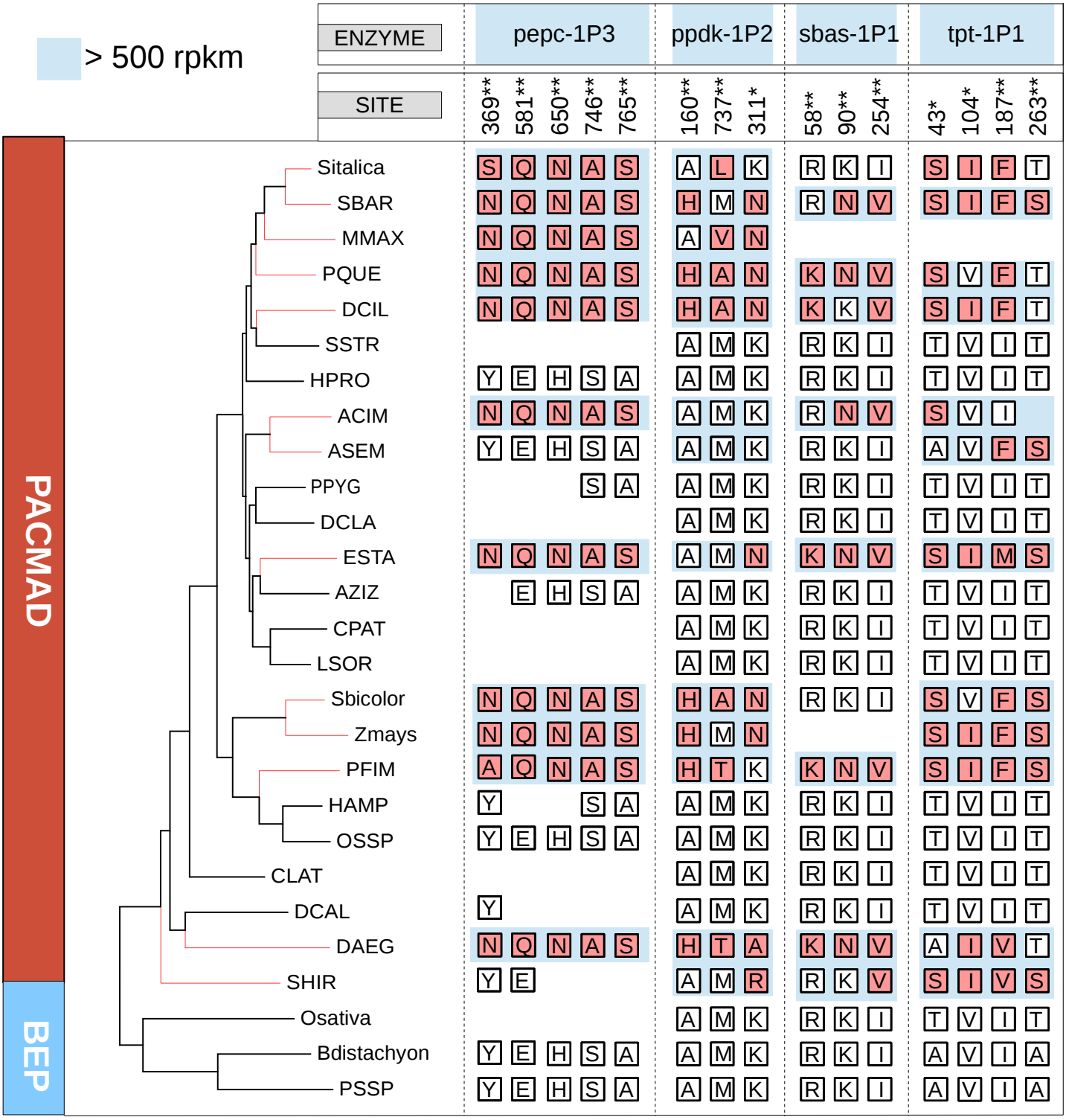
[1] df = degrees of freedom. For each variable, the degrees of freedom for the residuals are given after the comma.


Setaria italica
Setaria barbata
Megathyrsus maximus
Panicum queenslandicum
Digitaria ciliaris
Sacciolepis striata
Homopholis proluta
Alloteropsis cimicina
Alloteropsis semialata
Panicum pygmaeum
Dichanthelium clandestinum
Echinochloa stagnina
Acroceras zizanioides
Cyrtococcum patens
Lasiacis sorghoidea
Sorghum bicolor
Zea mays
Paspalum fimbriatum
Hymenachne amplexicaulis
Steinchisma sp.
Chasmanthium latifolium
Danthonia californica
Dactyloctenium aegyptium
Stipagrostis hirtigluma
Oryza sativa
Brachypodium distachyon
Poa sp.
Glycine max
Populus trichocarpa
Arabidopsis thaliana
10*
9*
8*
7*
6*
5*
4*
3*
2*
1*
Paniceae
PACMAD
BEP
0.1


BRANCHES
C4
Non-C4
OSSP
SHIR
DAEG
DCAL
CLAT
OSSP
HAMP
PFIM
Zmays
Sbicol
LSOR
CPAT
AZIZ
ESTA
DCLA
PPYG
ASEM
ACIM
HPRO
SSTR
DCIL
PQUE
MMAX
SBAR
Sitalica
RPKM
aspat-3P4
1200
500
βca-2P3
25000
500
nadpme-1P4
6000
500
pck-1P1
4000
500
ppc-1P3
6000
500
ppc-1P6
8000
500
ppdk-1P2
7000
500
ppdkrp-1P1
400
sbas-1P1
4000
500
tpt-1P1
2000
500


LEAF EXPRESSION
0
> 500 rpkm
non-C4
C4
CO-OPTED
LEAF/ROOT RATIO
0
> 50
non-C4
C4


TIMES CO-OPTED
ANCESTRAL EXPRESSION (rpkm)
10
8
6
4
2
0
100
200
300
400
ENZYME
AK
ALA-AT
ASP-AT
DIT
NADP-MDH
NADP-ME
NDH
PCK
PEP-CK
PPa
PEPC
PPDK
SBAS
TDT
TPT


> 500 rpkm
ENZYME
pepc-1P3
ppdk-1P2
sbas-1P1
tpt-1P1
SITE
369**
581**
650**
746**
765**
160**
737**
311*
58**
90**
254**
43*
104*
187**
263**
PACMAD
BEP
Sitalica
SBAR
MMAX
PQUE
DCIL
SSTR
HPRO
ACIM
ASEM
PPYG
DCLA
ESTA
AZIZ
CPAT
LSOR
Sbicolor
Zmays
PFIM
HAMP
OSSP
CLAT
DCAL
DAEG
SHIR
Osativa
Bdistachyon
PSSP